REMARKS

Prior to Applicants' March 20, 2002 Amendment Under 37 C.F.R. §1.115, claims 60-145 were pending in this application. New claims 146-200 were added by their March 20, 2002 Amendment. Claims 60-145 have been canceled hereinabove in order to clarify the status of the present pending claims, namely 146-200. Accordingly, and as in the case of Applicants' March 20, 2002 Amendment, claims 146-200 are once again being presented for further examination in this application.

Amendments to Specification

For the sake of accuracy and completeness, a number of citations and informalities have been corrected in the specification. These corrections affect pages 2, 4, 6, 22, 33 and 34. Replacement specification pages are attached to this paper as Exhibit 1. Marked-up versions of these pages are attached as Exhibit 2.

These minor errors in various citations came to the attention of Applicants' undersigned attorney while preparing the Information Disclosure Statement that was filed on January 22, 2003. The informalities in the form of mispellings or typographical errors came to light while proofreading these five pages. Thus, the word "fluoresceinated" (page 6), "incorporated" (page 34), and "creating" (page 34) have been corrected as shown in the marked-up versions (Exhibit 2).

Entry of the amendments to the specification pages is respectfully requested.

Cancellation of Previous Claims

As indicated in the opening remarks above, Applicants added new claims 146-200 in their March 20, 2002 Amendment, while intending to cancel the previously pending claims, 60-145. Because the instructions in their March 20, 2002 Amendment did not specifically direct the cancellation of claims 60-145, Applicants have directed the cancellation above. By so doing, it has been made clear that only claims 146-200 are pending and under examination in this application. Any inconvenience caused by this oversight in Applicants' March 20, 2002 Amendment is sincerely regretted.

The cancellation of claims 60-145 is respectfully requested.

Favorable action on this application is respectfully urged.

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SUMMARY AND CONCLUSIONS

Claims 146-200 are pending and continue to be presented for examination. To correct an oversight in Applicants' previous amendment filed on March 20, 2002, claims 60-145 have been canceled.

No fee or fees are believed due in connection with this paper. In the event that any fee(s) are due, however, The Patent and Trademark Office is hereby authorized to charge the amount of any such fee or fees to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney request that he be contacted at the number provided below.

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various methods to introduce sequences into a PCR amplicon, such that the final product would have self-complementary sequences at each end (U.S. patents #5,439,793, #5,595,891, and #5,612,199, each of which is incorporated herein by reference).

Both the original PCR amplification system and various improved PCR systems suffer from the limitation of a necessity for expensive dedicated thermocyclers to provide the multiple temperature conditions that are intrinsic to the PCR method. This necessity is derived from the problem that the extension of a primer creates a product that has a stronger association with a template that the primer used to create it. As such, in a system like PCR, temperatures that allow binding of a primer are too low to allow separation of the extended product from its template and temperatures that are elevated enough to allow the separation of the extended product are too high to allow another priming event. The second priming event cannot take place until after the first extended strand is separated from its template. As such, in PCR amplification, primer binding to template and the sequential release of the extended primers from the template have to be carried out at separate distinct temperatures and require a thermocycler to provide repeated sequences of distinct thermal steps. The existence of discrete cycles with different conditions also necessitates an optimization of temperature for each individual temperature step as well as an appropriate timing for each step. Similar problems also apply when ligation is used in the LCR reaction (Backman, K. et al. European Patent [~~Application~~] Publication No. 0 320 308 **B1**, Landegren, U., et al., 1988 Science 241; 1077, Wu, D. and Wallace, R.B. 1989 Genomics 4; 560, Barany, F. 1991 Proc, Nat. Acad. Sci. USA 88; 189) where the temperature required for binding individual probes is less than the temperature required to release them after they have been stabilized by a ligation event. All of the foregoing documents are incorporated herein by reference.

Others have recognized these limitations and tried to overcome them by providing means to accomplish multiple cycles under isothermal conditons. Examples of this are 3SR (Kwoh, D.Y. et al., **(1989)** Proc. Nat. Acad. Sci. USA 86; 1173-1177) and NASBA (Kievits, T. et al., 1991 J. Virol. Methods 35; 273-286, the contents of each of which is incorporated herein by reference). Each of the preceding systems has the limitation of a necessity for the introduction of an RNA promoter into the structure of the nucleic acid being amplified. Consequently, there is also a limitation that these systems are dependent upon a cycling reaction between DNA and RNA forms of the sequence of interest. A dependency upon the

retaining the other two limitations of a need for a restriction enzyme and modified substrates.

Temperatures used for the various steps of full cycle amplification are dictated by the physical constraints that are intrinsic to each step. As such, in prior art the temperature used for complete displacement of extended strands from templates is typically around 92-95°C. This high temperature has been used to insure an adequate efficiency of separation such that an extended strand can be used as a template for subsequent reactions. When PCR was first described, the polymerase was derived from E. coli and as such there was essentially complete thermal inactivation of the polymerase after each denaturation step that required the addition of more enzyme (Saiki et al., 1985 230; 1350-1354; **cited supra**). This problem was addressed by the use of a DNA polymerase from a thermophlilic bacterium, T. aquaticus, in PCR reactions (Saiki et al., 1988 Science 239; 487-491). Each of the foregoing Saiki publications is incorporated herein by reference. Due to its inherent heat stability, enzyme was continuously present throughout the PCR cycles and no further additions were required. Since that time, polymerases from other thermophiles have also been isolated and used in full cycle reactions. However, even though they are more robust in their resistance to thermal inactivation, these polymerases all suffer from a limitation of having a certain level of inactivation after each denaturation step that is dictated by a half-life for that particular enzyme at the temperature used for denaturation. Also the high denaturation temperature can also decrease the levels of dNTP substrates by hydrolysis and lead to inactivation of proteins that may be added to supplement the efficiency or specificity of the reaction.

Full cycle PCR conditions have been modified such that lower denaturation temperatures could be used Auer et al., (1996, Nucl. Acids Res 24; 5021-5025, incorporated herein by reference) have described a procedure that used dITP, a natural neutral analogue of dGTP. By this substitution, they succeeded in avoiding amplification of double-stranded DNA that may be present in their samples and only amplified RNA targets. By no means is there recognition or appreciation of a utility for DNA targets. In fact, they teach away since their purpose is to avoid the use of DNA targets as templates. Their teachings have a limitation that the substitution dITP also necessitated a compensatory decrease in the temperatures used for the annealing (50°C). In addition, the art described by Auer et al. relies upon the use of a nucleotide analogue that is known for a lack of discrimination for base pairing, thereby introducing the possibility of random variations being

However, primer labeling has the limitation that there can be secondary structure or problematic sequences in the template strand that can cause inappropriate chain termination events that create ambiguities in the proper base assignment for that position. Incorporation of labeled dNTPs during the extension of the primer also suffers from this limitation. This limitation is valid regardless of whether radioactive or non-radioactive labels are used.

This limitation has been circumvented by the choice of the chain terminator nucleotide itself as the source of the label. This has been described by Hobbs and Cocuzza in US patent # 5,047,519 and by Middendorf et al., in U.S. Patent # 4,729,947 for fluorescently labeled ddNTPs and by Middendorf et al., in U.S. Patent # 4,729,947 for biotin labeled ddNTPs that were later marked by fluorescent avidin. (For further reference refer to U.S. patent nos. 5,027,880; 5,346,603; 5,230,781; 5,360,523; and 5,171,534.) Each of the foregoing seven patents are incorporated by reference into this application. By this method, signals will be generated by strands that have incorporated a chain terminator. The presence of strands that have been terminated without the incorporated of a terminator nucleotide is now irrelevant since they are incapable of signal generation. However, this method has the limitation that the presence of additional chemical groups that provide signal generation produce steric or other inhibitory problems for the polymerase directed incorporation of the labeled terminator nucleotide, thereby decreasing the efficiency of the reaction (Prober et al. in U.S. Patent # 5,332,666, incorporated herein). It has also been suggested that biotinylated dideoxynucleotides could be used to provide signal generation, but these modified terminators were predicted to share the same limitations as their [~~fluorescenated~~] **fluoresceinated** counterparts, i.e. difficulty in corporation by most commonly used polymerases (S. Beck 1990 Methods in Enzymology 184; [~~611~~] **612-617**, also incorporated herein). Some compensation for this inefficiency of incorporation can be achieved by increasing the amounts of polymerase in the reaction and/or by increasing the amounts of template DNA being copied. These compensatory steps suffer the limitation of increased costs associated with higher amounts of an expensive enzyme, DNA polymerase, or with preparation of adequate amounts of high quality template

art has indicated that the association and dissociation of short complementary oligonucleotides occurs as an equilibrium reaction whose characteristics are determined by the temperature, salt conditions, base content and length of the complementary sequences. The influence of these factors has been reviewed by J.G. [~~Wechsler~~] ~~Wetmur~~ ([1991] Crit. Rev. Biochem. Mol. Biol. 26; 227-259, incorporated herein). Although larger strands of complementary DNA exist as double-stranded molecules in stable configurations that do not readily dissociate over a wide range of conditions, it is well known that they do form temporary and localized relaxations of interstrand bonding. The term "breathing" has been used to describe this localized disruption of hydrogen bonding. A pathway for "breathing" to create two-dimensional structures in double-stranded DNA molecules that contain palindromic sequences has been described by A. Kornberg and T. A. Baker in "DNA Replication, 2nd Edition" (1992) W. H. Freeman and Co. NY, NY., ~~pages 44-46;~~ the contents of which are incorporated herein by reference.

In the present invention, as described above, the transition of a segment of a linear double-stranded molecule to an intra-strand stem-loop structure can allow primer initiation events to take place prior to separation of an extended primer from its template. The equilibrium between these two structures is dependent upon a number of factors. First, for successful primer binding, the segment of the initial primer that binds to the target must be of appropriate length and base composition so as to allow stable priming at the temperature being used for the reaction. Second, the segment of the primer that participates in self-hybridization after an extension of the initial primer must be of appropriate length and base composition such that a partial dissociation of the extended primer from the template can allow the formation of a sufficiently stable secondary structure, i.e., the stem of a stem-loop structure.

Temperatures appropriate for these reactions are below those that would be required for separation of an extended primer from its template. In an isostatic reaction, a single temperature can be used for binding, extension and secondary structure formation. Or if so desired, limited cycling conditions can be used where different temperatures are used to optimize these events. The use of different temperatures for limited cycling may be useful for primer binding, primer extension or a localized separation of some of an extended product from its template. The temperatures being used for any and all of these steps should also be appropriate for the particular polymerase being used in the reactions.

site for the novel primer. As a result of this, each strand is capable of forming a stem-loop structure at one end of the amplicon. The exposure of the primer binding site in the single-stranded loop structure can then generate a further series of primer binding and displacement reactions by the same process previously depicted in Figure 1, thereby allowing generation of non-linear amplification of the sequences of interest under isostatic or limited cycle conditions. This product is different than that created by Rose et al. by non-linear amplification since their processes led to the extended sequences always being located between self-complementary regions whereas in this aspect of the present invention, the extended sequences are outside of the stem-loop regions. In addition, the processes of this aspect of the present invention regenerate a binding site by secondary structure formation whereas in Rose et al., the binding site is in the stem region of a potential stem-loop structure and is never available for another binding event without denaturation of the amplification product.

Primer sequences appropriate for carrying out this aspect of the present invention are dependent upon the factors described previously for linear amplification. The segment of the primer that binds to the target must be of appropriate length and base composition in order to allow stable priming at the temperature being used for the reaction. The segment of the primer that participates in self-hybridization after an extension of the primer must be of appropriate length and base composition such that a partial dissociation of the extended primer from the template is sufficient for the creation of a stable secondary structure, i.e., the stem of a stem-loop structure. This structure does not have to be permanent but only sufficiently stable such that it can allow another priming event. In addition, this aspect of the invention involves the creation of a complementary copy of the stem-loop sequences of the extended novel primer. This necessitates that the segment of the primer that participates in self-hybridization after an extension of the primer must be of appropriate length and base composition such that the sequences involved in secondary structure can still be used as templates. In addition to base composition and length, stability of primary and secondary structures can be influenced by the incorporation of modified bases into the primers, the extended sequences or both. These can either raise or lower the Tm of the segments where they are present. An example of a modification of a base that can raise the Tm of a segment can be but is not limited to the addition of an Ethidium Bromide moiety as described in [~~ENZ XX~~] ~~EP 0 231 495 B1~~. An example of a modification of a base that can decrease the Tm of a segment can be

but is not limited to the use of Inosine as described by Auer et al., (1996, Nucl. Acids Res. 24; 5021-5025, **cited supra,** contents already **[~~incorporation~~] incorporated** herein).

A non-linear amplification product can also be synthesized under isostatic or limited cycle conditions by a novel nucleic acid construct that comprises two first segments and one second segment. Each of the first segments is complementary to a strand of a nucleic acid or its complement and the second segment is capable of forming a secondary structure after extension of one of the first segments. This construct would be capable of **[~~erating~~] creating** a product that has a pair of complementary potential stem-loop structures. This product could be formed by a continuous series of the following steps. One first segment and one second segment of the novel construct could carry out the same continuous series of binding, extension, secondary structure formation, regeneration of a primer binding site, second binding, second extension and strand separation steps that have been described previously for linear amplification by a single novel primer. In addition, the product of this synthesis could be used as a template for a series of binding and extension steps by the other first segment as had been described above for non-linear amplification with a novel primer and a standard primer. A potential series of different forms that these steps could generate is given in Figures 5 and 6. The series of events that this novel construct can potentially carry out is the same as described previously and the final product shown in Figure 6 is the topological equivalent of the final product of Figure 2 with the two 5' ends of the primers bound together.

A non-linear amplification product can be synthesized by the use of two novel primers that are complementary to different strands of a target nucleic acid by a continuous series of the following steps under isostatic or limited cycle conditions. Novel primer (A) binds to a target strand and there is the same series of extension, secondary structure formation, regeneration of a primer binding site, second binding, second extension and separation of the extended primer from the template as described previously for linear amplification with a single novel primer. As extended novel primers are displaced by binding and extension of other novel primers, these single-stranded products can bind novel primer (B) and allow it to be extended to create a full double-stranded amplicon. This potential series of events is depicted in Figure 3. As described previously, the formation of the complement of an extended displaced primer creates a template with secondary structure that should allow multiple binding, extension and displacement events under isostatic or limited cycle conditions. A product can be formed that has secondary structure at